Estimating the Population Level Effects of Viral Coinfection in a Model *Drosophila* System

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AUTHORIZATION TO SUBMIT THESIS

This thesis of Johnathan A. Kaiser, submitted for the degree of Master of Science with a Major in Biology and titled "Estimating the Population Level Effects of Viral Coinfection in a Model *Drosophila* System," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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ABSTRACT

Coinfection events, the simultaneous infection of hosts by multiple pathogens, may alter the course of disease(s) at the population level. Here, we utilize a system of *Drosophila* and the associated Drosophila C virus (DCV) and Drosophila X virus (DXV) to examine how viruses may interact to alter the dynamics of mortality and viral transmission. We find that coinfection reduced the effects of the viruses on host mortality. The main effect of these viruses appears to be on greatly reducing expected variance in mortality between small populations exposed to the viral treatments rather than on cumulative mortality between populations over experimental time. We also show that the effects of coinfection on transmission rates and infection severity are non-additive in small populations exposed to a single infectious donor. These studies provide a framework for the understanding and development of a tractable empirical system for modeling viral coinfection at the population level.

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INTRODUCTION

Pathogenic coinfection events, the simultaneous infection of hosts by multiple pathogens, are a common occurrence in natural populations (Susi et al. 2015, Cox 2001). In certain species and regions, it is thought that coinfection events may even be more common than the occurrence of relatively healthy individuals or those afflicted with a single infection (Petney & Andrews 1998). In coinfection cases the interaction between multiple pathogens can drastically alter the dynamics and course of disease. These interactions can result in a reduced net effect on the host as exemplified in increased survival rates of Ebola patients already harboring the malaria-causing *Plasmodium* parasite (Rosenke et al. 2016); or detrimental, through increased net effects such as decreased survival in individuals coinfected with human immunodeficiency virus (HIV) and hepatitis C virus (HCV) due to HCV-related cirrhosis (Pineda et al. 2005). Although the ability of coinfection to alter disease dynamics when compared to single infection is clear, there is still a gap in knowledge to fill regarding the magnitudes and implications of these changes on the population scale.

Drosophila is already an established model system for the study of innate immune responses with a swath of genetic tools and references (Hedges & Johnson 2008, van Rij et al. 2006). Couple this with the relative ease to use *Drosophila* in large numbers and there is great potential to scale this system for the study and modeling of virus-virus and virus-host dynamics from the molecular level to the population level. A *Drosophila*-virus system developed at the University of Idaho (UI) has already begun characterizing the host response in single viral infections of *Drosophila* with two distinct viruses: Drosophila C virus (DCV) and Drosophila X virus (DXV). DCV is a well-documented (+)ssRNA virus of the family *Dicistroviridae* and found in both natural and lab populations of *D. melanogaster* (Plus et al. 1975). Microinjection of DCV results in high virulence and near immediate systemic infection leading to near 100% host mortality within a week (Gomariz-Zilber & Poras 1995, Merkling & van Rijj 2015). Additionally, larvae and adult flies have both been documented as being infected via direct contact as well as oral/fecal environmental transmission (Gomariz-Zilber & Poras 1995). DXV is a more scarcely documented, bi-segmented dsRNA virus of the family *Birnaviridae*. DXV has been shown to result in CO_2 sensitivity in *D.*

melanogaster exhibited as mortality by hypoxia (Teninges et al. 1979). It has also been demonstrated that flies can pick up infection from extended incubation time with DXV-injected flies, however the exact route and time of transmission remains unclear (Teninges et al. 1979). Our research team at the UI has chosen these viruses for their ability to elicit different mechanisms of antiviral response in *Drosophila* and the capability of both viruses to be spread naturally in laboratory populations (Xu & Cherry 2013) (*table 1.0*). This model system is intended to be easily expanded upon (e.g., addition or substitution of viruses and host conditions) and available to the scientific community to be tested for generality across other prevalent arthropod borne virus systems that may be of major concern to human health efforts (Blanc & Gutiérrez 2015).

Population demographics such as survival are a key factor in modeling any epidemiological study, with mortality often acting as a proxy for virulence (Thomas-Orillard et al. 1995, Goldhill & Turner 2014, Gandon et al. 2001). Interplay between infecting pathogens can lead to synergistic, antagonistic, or moderate effects on not only net virulence, but on the virulence associated with each individual pathogen, and therefore is important to the individual evolutionary trajectories of pathogen genotypes (Alizon et al. 2013). Thus, understanding how coinfecting pathogens impact host demography is critical to the development of models based on observable outcomes.

Additionally, linking within host and between host pathogen dynamics remains a challenge in studying the spread of disease in any host-pathogen system at the population level (Susi et al. 2015). Many of the challenges with this connection stem from the difficulty in accurately quantifying transmission rates and magnitudes. Within the host, factors such as differences in individual host immune response may be altered by the presence of multiple pathogens (Murphy et al. 2013). Similar to the effects on host demography by coinfection, a primary pathogen can confer immune resistance against later infections (DaPalma et al. 2010). The opposite effect is also possible in that certain viral infections can suppress the host immune system and lead to additional opportunistic coinfection events. These factors may alter the ability of a pathogen to replicate within host and in turn affect pathogenic shedding patterns. Between hosts, patterns of transmission may vary based on primary routes of transmission and whether they are environmentally or directly transmitted and in what manner (e.g., airborne, oral/fecal, waterborne, etc.).

This thesis describes two studies regarding viral coinfection events utilizing the *Drosophila*-virus system developed at the UI. These studies address how two viruses (DCV and DXV) interact, if at all, to alter the population level dynamics of mortality and transmission. The first study examines the effects on adult *Drosophila melanogaster* mortality through an extended time course of 35 days following exposure to viral treatments through oral infection. The second study addresses the effects of coinfection by DCV and DXV on viral transmission rates among adults and severity of infection with viral exposure occurring through a single adult fly in a small population.

Table 1.0. *Adapted from Xu & Cherry 2014*. Comparison of study viruses DCV and DXV and host response. Both are potentially lethal arthropod specific RNA viruses with similar sites of infection. These viruses differ in the antiviral response elicited, with DCV activating the JAK/STAT pathway and DXV activating the Toll pathway in *Drosophila melanogaster*.

Virus	Host Range	Family	Genome	Segments	Pathogenesis	Site of Infection	Antiviral Response
Drosophila C Virus (DCV)	**Insects**	***Dicistroviridae***	**(+)ssRNA**	**1**	**Potentially lethal**	**Fat body, periovarial sheath, tracheae, muscles, digestive tract**	**JAK/STAT, RNAi,autophagy, transcriptional pausing**
Drosophila X Virus (DXV)	**Insects**	***Birnaviridae***	**dsRNA**	**2**	**Anoxia, Non-lethal or late onset mortality**	**Fat body, tracheae, muscles, digestive tract, ovaries**	**Toll pathway, RNAi**

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Chapter 1

INFLUENCE OF VIRAL COINFECTION ON *DROSOPHILA MELANOGASTER* MORTALITY UPON ORAL INFECTION BY DROSOPHILA C VIRUS (DCV) AND DROSOPHILA X VIRUS (DXV)

ABSTRACT

The characterization of variation in host demographic properties such as mortality rate is crucial to understanding the impacts of viral infection on the population level. Interactions between coinfecting viruses may act antagonistically or synergistically to alter the relative virulence of each virus in a host. Using *Drosophila melanogaster* and two of its associated viruses, we compared daily mortality counts across three viral treatments for a period of five weeks in orally infected adult fly populations. The viral treatments, Drosophila C virus (DCV), Drosophila X virus (DXV), and Coinfection (COI) were compared to a mock-infected control line. We analyzed results using a hierarchical beta-binomial model with maximum likelihood estimation. We find that there appears to be little variation in cumulative mortality between viral treatments. However, the variance in mortality amongst replicate vials (within treatments) appears to be strongly affected by viral treatment. Results from all analyses suggest a clear non-additive effect of DCV and DXV coinfection on mortality in *D. melanogaster*.

INTRODUCTION

In an increasingly globalized society, pathogen interactivity is becoming a major area of study with an acknowledgment that coinfection events are likely to be the norm rather than the exception (DaPalma et al. 2010). Interactions between pathogens within a shared host may drastically alter the dynamics of infection and disease outcome for both the pathogens and the host by affecting disease severity, duration, and transmission (Chertow et al. 2013, Pineda et al. 2005, Susi et al. 2015). There is a need to generate biological and mathematical models for pathogen interactivity to better understand and effectively respond to disease outbreak in natural populations. A critical step towards generation of these models is to elucidate the impacts of coinfection on population demographic properties such as mortality. A better understanding of host demographic properties will shed light on both short-term and long-term impacts of exposure to multiple pathogens. For example, the spread of viral disease can be stunted by pathogenic induction of high host mortality rates and low fecundity (Keeling & Rohani 2008).

Utilizing a host-virus system of *Drosophila melanogaster* and the associated Drosophila C virus (DCV) and Drosophila X virus (DXV), this study explores the effects of viral coinfection events on host mortality. DCV is a common virus, often used to model innate immunity and antiviral responses in the *Drosophila* system (Chtarbanova et al. 2014). DCV results in high virulence and near immediate systemic infection generally leading to 100% host mortality within a week of viral injection (Gomariz-Zilber et al. 1995, Merkling & van Rij 2015). This study, however, is based on infection through an oral inoculation protocol meant to more closely resemble natural routes of infection when compared to viral injection (Bonning & Miller 2010, Merkling & van Rij 2015). While it is possible for oral inoculation by DCV to result in systemic infection, it is more often associated with a local immune response (Ferreia et al. 2014). Additionally, oral inoculation often results in a milder infection associated with low rates of mortality (Durdevic et al. 2013, Goic et al. 2013, Ferreia et al. 2014). Although the effects and pathology of DXV are not as well understood as DCV in *Drosophila*, it is still utilized in the study of the innate immune system and has contributed to knowledge regarding the *Drosophila* RNA-interference (RNAi) response (Zambon et al. 2005). The JAK/STAT pathway and the RNAi response are

generally regarded as the primary *Drosophila* antiviral responses (Ulvila et al. 2010, Avadhanula etl al. 2009, Zambon et al. 2005, Costa et al. 2009, Dostert et al. 2005). Additionally, inoculation by DXV has been shown to induce anoxia sensitivity, exhibited by increased rates of mortality (Teninges et al. 1979).

This study addresses questions regarding the effect of oral infection on *D. melanogaster* mortality across three different viral treatments (DCV, DXV, COI) over an extended time course of 35 days. More specifically, we seek to answer the following questions: How does exposure to DCV affect the rate of mortality in *D. melanogaster* compared to mock-infected lines? How does exposure to DXV affect the rate of mortality in *D. melanogaster* when compared to a mock-infected line? And how do possible interactions by DCV and DXV change the rates of mortality in *D. melanogaster* compared to single viral exposure events?

MATERIALS & METHODS

Fly and virus stocks

All flies used in the mortality study were of the Oregon R WT^{+} strain of *Drosophila melanogaster* treated for viral and bacterial infection via tetracycline and egg bleaching (Merkling & van Rij 2015). Flies were incubated in a Genesee Scientific I Drosophila incubator (model 59-400LH) on 12-hour night/day cycles at 25°C and at 65% relative humidity. Fly stocks were separated into four groups reared in weekly increments. Each group was reset monthly by random sorting of five males and five females per vial from the current two-week old stock population to form the new group. All experimental flies were age controlled from the same stock group.

Two viruses were utilized in this study, Drosophila C virus (DCV) and Drosophila X virus (DXV). DCV is a single stranded RNA virus, 9,264 base pairs in length, and a member of the family *Dicistroviridae* family. We used the Charolles strain of DCV that was isolated from a laboratory population of *Drosophila* in 1972 (Jousset et al. 1972) and that we obtained from Dr. Luis Teixeira (Instituto Gulbenkian de Ciencia). DXV is a bi-segmented, double-stranded RNA virus, 6,603 base pairs in length, and a member of the family *Birnaviridae* (Teninges et al. 1979, Nagy & Dobos 1984). DXV was originally isolated in

1979 as a contaminant in studies involving Sigma virus in *Drosophila melanogaster* (Teninges et al. 1979). We obtained our DXV stock from Dr. Louisa Wu (University of Maryland). Both DCV and DXV were cultured in Schneider's Drosophila Line 2 (S2) cells and titrated to a tissue culture 50% infectious dose ($TCID_{50}$) of $2x10^9$ units/mL. This is the endpoint dilution of virus required to cause cytopathic effects in 50% of inoculated cell cultures. Viral stocks were kept in 50µL aliquots at -80ºC.

Oral infection & measuring mortality

Experimental flies were age controlled by dumping adults from stocks and subsequently collecting newly emerged flies within a 24-hour window. Newly emerged flies were then sexed and collected in vials at roughly a 1:1 ratio of males to females and aged to maturity for 72 hours. Mature flies were starved for a four-hour period and then transferred to vials with viral feeding medium. Viral feeding medium was composed of 225µL of viral stock suspended in 225µL of S2 media, 50µL 25% sucrose and 10µL red food dye. Feeding tubes contained 100µL of viral feeding medium spread on a 1.91cm diameter circular cut of Whatman® filter paper (cat No 1001 150). Flies were left in feeding tubes for six hours and then sorted by visual confirmation of recent feeding by red dye seen in midgut through the exoskeleton on the ventral side of the abdomen.

Following oral inoculation, flies were sorted into vials with fresh food medium. Each treatment (Mock, DCV, DXV, COI) consisted of 10 replicate vials with 10 flies each for a total of 100 flies per treatment. Each replicate vial began with a 1:1 male to female ratio (5♂ & 5♀). Every seven days, flies were tipped into new vials with fresh food medium to maintain the experimental group as well as to avoid the emergence of offspring into the experimental population. All experimental vials were stored in identical conditions to fly stocks on a 12-hour day/night cycle at 25°C and at 65% relative humidity in a separate incubator.

Mortality was scored daily as a count of newly deceased flies in each vial over the course of 35 days. Records were kept of deceased flies as well as the number and sex ratio of remaining flies in each vial. On occasion, pupae were transferred with experimental organisms and would emerge, resulting in an increase in the population of the replicate vial. Additionally, the occasional experimental fly was lost during the transfer process, resulting

in a population decrease not due to a mortality event. Neither event was prevalent but both did occur on more than one occasion and are taken in to account in analysis via a hierarchical beta-binomial model.

Deceased flies were not collected and assayed for viral presence due to complications in timely retrieval. Flies were housed in small populations that would require anesthetization prior to collection of deceased individuals. Adult flies infected with DXV have been shown to exhibit greater sensitivity to anoxia-induced mortality by CO_2 exposure (Huszar & Imler 2008). Due to this DXV induced CO_2 anoxia, all post infection anesthetization was conducted on ice. However, anesthetization processes in general have been associated with altered demographic properties in *Drosophila simulans* and *Drosophila melanogaster* (Champion De Crespigny & Wedell 2008). As such, deceased flies were not collected immediately following a mortality event to avoid potentially excessive stress from repeated anesthetization of experimental populations nor did anesthetization occur during transfers. Transfers were done by tipping flies from week-old vials to a newly prepared food vial. Deceased flies were removed from the populations at the weekly food vial transfers every seven days but not collected due to corpses being decomposed, eaten, or buried deep within the food medium.

Modeling mortality & data analysis

Hierarchical Beta-Binomial Model with Maximum Likelihood Estimation (MLE)

For the hierarchical model with MLE, data were first aggregated into 5 time periods corresponding to experimental weeks: days 1 - 7, days 8 - 14, days 15 - 21, days 22 - 28, and days 28 - 35. This grouping of time intervals was chosen due to the weekly intervals at which flies were transferred to new vials. Observation of raw mortality (*fig. 1.1*) over days shows that within a week there is a tendency for the response (mortality) to remain low across replicates until the end of the weekly intervals. Aggregating days into weekly periods allows for comparison focused on variation over time due to the treatments without over estimating the effect of daily variation stemming from experimental design. Within a given treatment (Mock, DCV, DXV or COI) three sets of random variables were defined for analysis of the data set as:

- $Y_{i,t}$: random response variables connected with the mortality of experimental flies in replicate i during the period t, where $i = 1, \ldots, K$ and $t = 1, \ldots, 5$.
- $E_{i,t}$: random variables associated with emergent pupae in replicate i during period t, with the same index sets for i and t as for $Y_{i,t}$.
- $D_{i,t}$: random variables associated with number of non-mortality experimental fly losses during transfer or otherwise in replicate i during period t, with the same index sets for i and t as for $Y_{i,t}$.

It should be noted that all random variables are counts and are thus non-negative integers. Additionally, because non-mortality related losses or additions to the experimental populations were rare, the vast majority of values for variables $E_{i,t}$ and $D_{i,t}$ are 0. Due to the predominance of 0 values these variables, although technically random for each replicate, are taken as fixed numbers (observations) as there is not enough information to model these effects.

Distributions were considered binomial for $Y_{i,t}$ for each time period. That is, with $N_{i,t}$ flies alive in replicate i at the beginning of period t, the number of mortalities $Y_{i,t}$ are binomial random variables with a distribution of conditional probabilities of mortality $\theta_{i,t}$. This parameter $\theta_{i,t}$ is a probability that can be viewed as the "force of mortality" within replicate i over the time interval t. In other words, $\theta_{i,t}$ accounts for the complex of mechanisms of mortality consisting of the entire unknown set of reactions (including but not restricted to chemical, biological, environmental, and physiological) within a given replicate vial i during a given time-interval t.

Observation of the cumulative mortality in each individual replicate (*fig. 1.1*) over study time confirms the intuitive conclusion that there is an apparent level of variability across replicates within a given treatment. This implies that the conditional probabilities of mortality $\theta_{i,t}$, are different for each replicate within a treatment. Modeling this variability in $\theta_{i,t}$ is accomplished using a hierarchical structure in which those conditional bionomial probabilites are modeled as random variables following their own beta distributions with parameters α_t and β_t (Williams 1975). The best-fit model by MLE contains two beta distributions, one for the first two-week period post infection, and one for weeks three

through five (see below). These distributions consist of parameters (α_1, β_1) and (α_2, β_2) respectively.

To summarize, in this model the parameter $\theta_{i,t}$ controls the distribution of $Y_{i,t}$ and represents unobservable random effects across replicate vials (i) during period (t) with estimation and testing focused on variables α_t and β_t which control the distribution of $Y_{i,t}$. Testing between models with different numbers of restrictions used to determine the number of distinct (α_t, β_t) pairs needed to account for variability among replicates within each treatment was accomplished through likelihood ratio tests. The outcome of this procedure was to use one beta distribution to represent the values $\theta_{i,t}$ in time periods 1 and 2, and a second beta distribution in time periods 3, 4, and 5. We denote the parameters of these 2 different beta distributions as (α_1 , β_1) and (α_2 , β_2). Estimation of parameters (α_j, β_j); j = 1, 2 was done through maximization of the marginal log likelihood of (α_j, β_j) given the observed value $\boldsymbol{y}$.

RESULTS

General effect of viral infection on cumulative mortality

Raw cumulative counts of mortality across all treatments over a span of 35 days show marginally increased levels of mortality in all three viral treatments (DCV, DXV, COI) compared to the Mock line. All viral treatments and the Mock line show between 60% and 80% mortality across replicate vials as well as exhibit similar trends with mortality increasing in a weekly stepwise fashion 2 to 3 days before vial transfers (*fig. 1.2*). Mortality in DCV can be observed rising above all other treatments early in the experiment (DPI five through 20) with DXV mortality greatly increasing near the beginning of week three (~ DPI 18). Mortality across the COI treatment remains low throughout most of the experimental time, however it rises to near 80% mortality by DPI 35. Despite ending with the highest proportion of deceased flies, the patterns of mortality exhibited by the COI treatment across time more closely resemble the Mock line than either DCV or DXV.

The observed stepwise patterns in mortality are likely an artefact of the week-long timespan between transfers and larval density. Newly hatched larvae churn up the food medium within vials causing the substance to become soupy and sticky at high larval

density. This was not an uncommon occurrence and affected replicate vials across all treatments. Unpublished work by Gonzalez et al., in which flies were housed in isolation and transferred to new vials in four-day intervals, did not exhibit the same stepwise pattern of mortality. By analyzing our mortality data accumulated over weekly intervals we minimize the noise in our dataset due to variation in mortality associated with the number of days after each weekly transfer event.

Hierarchical beta-binomial model using maximum likelihood estimation MLE

Likelihood ratio tests of the Mock line versus viral treatments across both beta distributions provide strong evidence for a difference between the DCV and Mock treatments ($T^* = 9.1957$, $P = 0.0101$), weak evidence for an effect when comparing DXV and Mock ($T^* = 5.1595$, $P = 0.0758$), and no evidential difference between Coinfection (COI) and the Mock line ($T^* = 3.9752$, $P = 0.1370$) (*table 1.1*).

Likelihood ratio tests were also conducted on all pairwise combinations of viral treatments, again across both distributions. These results provide strong evidence for a difference between DCV and COI ($T^* = 6.5098$, $P = 0.0386$). However, there is no evidence for a difference between any other pairwise combinations of viral treatments (*table 1.2*). Overall, the results from *table 1.2* reinforce the trends reported in *table 1.1*. Interestingly, we found no evidence for a difference between DCV and DXV ($T^* = 3.5152$, $P = 0.1724$). DXV treated lines do not behave drastically differently from either the Mock line or the DCV line despite strong evidence for differing behavior of the DCV and Mock lines. This warrants a closer examination of the estimated models for treatments DCV and DXV.

Interpretation of parameter estimates for the model applied to the DCV and DXV treatments is accomplished by a transformation of α and β to produce two new parameter estimates, μ_j and ϕ_j, where μ_j is the expected mean value of the beta distribution and ϕ_j is directly proportional to the variance within the distribution. The subscript $j = 1$ is associated with the first distribution across weeks one and two and $j = 2$ is associated with the second distribution for weeks three through five. Parameter estimates for fits of the model to individual treatments suggest differences among treatments are reflected more in variances ϕ_j, than in mean values μ_j (*table 1.3*). The COI treatment is predicted to behave reasonably similarly to the Mock treatment with a slight, but non-significant (likelihood ratio test, $T^* =$

3.9752, P = 0.1370) increase in expected mortality. DCV exhibits a stark contrast to control treatments and the main effect of the viral treatment appears to be in nearly eliminating the variability in conditional mortality probabilities across replicate vials. Interestingly, we find that DXV treated lines appear more similar to the Mock and COI treatments within the first two-week period (μ_1, Φ_1), but shift to greater similarity with DCV treated lines in the final three-week time period (μ_2, Φ_2). A plot of the estimated cumulative density functions based on *table 1.3* for the first two weeks of the study and composing the first distribution is presented in *figure 1.3*, and for the last three weeks and second distribution in *figure 1.4*. A dramatic reduction in variability for the DCV treatment is seen in both the early and late time periods as the associated beta distributions accumulate probability at nearly a single point. This implies that the values for conditional probabilities of mortality ($\theta_{i,t}$) are nearly identical across all replicates in the DCV treatment within a time interval. The same is true of the DXV treatment in the later distribution associated with weeks three through five (*fig. 1.4*).

DISCUSSION

Our data show that, while there is no major difference between the overall probabilities of mortality across treatments, there is an effect on the variance of the probability of mortality among replicate vials within a given treatment. Additionally, when considering the variance within treatments, the COI lines behave most similarly to the Mock lines by consistently exhibiting high variance amongst replicate vials. The single infection lines of DCV and DXV both see greatly diminished variance in mortality probability across replicates over at least one of the two distributions modeled. This is evidence for a non-additive effect on host mortality in *D. melanogaster* when exposed to DCV and DXV in conjunction through oral infection.

While there is not a clear difference regarding the central tendencies of the beta distributions (μ_j) between treatments, there does appear to be a definitive difference in the variability of distributions (Φ_j). Mock lines and the COI treatment tend to have the most similarity overall in the distributions of conditional probability of mortality. What is perhaps surprising is that these lines exhibit the highest rates of variance in mortality probabilities

between replicates. This can be explained by unknown competing risks of mortality inherent in any population. It is not possible to control for all possible risks of mortality (biological, physiological, behavioral, environmental, etc.) and that is shown in the high variance in mortality across replicates in the Mock treatment. There is evidence that there is almost no variance in conditional mortality probabilities among replicates in DCV treated lines, with values of Φ_j very near 0 in both time periods, $j = 1, 2$. This can be interpreted as DCV overwhelming the unknown competing environmental, biological, or physiological risks to host mortality. This is not the same as the interpretation that DCV increases overall mortality within a population, but rather that when a mortality event occurs that it is most likely due to DCV. That is, the "force" of the virus has eliminated variability in mortality between replicates. We are confident in the assumption that DCV is the cause for reduced variation, as the effect we observe is consistent across all replicate vials of the treatment. The only constant between the replicates of this treatment that could differ from the other treatments is exposure to DCV.

DXV treated lines appear to have a behavioral duality, acting much more like the Mock and COI lines during the first two weeks of the experiment and then shifting towards reduced variance among replicates during weeks three through five. This result implies a tendency for late onset pathogenesis by oral infection of DXV exhibited as mortality. This may be due to a longer replication time for DXV or that the antiviral response against DXV is more effective than that of DCV. It is also possible that a small proportion of flies infected early on eventually shed and pass virus to other flies in the shared vial. This would result in higher proportions of flies infected per vial at later time points in the experiment. Regardless, DXV appears to follow in the trend of DCV throughout the second time-period of weeks three through five. Again, there is no apparent increase in overall rates or probabilities of mortality but the effect of DXV in later time points is to eliminate variation in the rates of mortality across replicates. The results of the present study suggest that when orally ingested, these viruses are not particularly lethal. This is in stark contrast to mortality studies involving DCV through injection in which DCV is highly lethal (Merkling & van Rij 2015). There is some support for injection versus ingestion related infection mortality with reduced variability in virulence of different DCV strains compared to the same strains infected via injection (Gravot et al. 2000).

Results from the hierarchical beta-binomial are also supported by a latent variable mixture model being developed by Ferguson et al. (in prep). This mixture model utilizes the same empirical data set on mortality, but focuses on estimating a rate of symptomatic individuals within a coinfected population based on demographic properties rather than characterizing the effect of the viruses on population level mortality. In brief, this is accomplished by treating empirical data as mixtures of individuals falling into different possible class states, i.e. asymptomatic (U) or symptomatic (S). A schematic of the latent variable mixture model is provided in *figure 1.5*. A baseline for the asymptomatic survival function $\Omega_U(t)$ is set using the Mock line. This information is then utilized to estimate the probability of individuals moving from class state U to S (P_s) in a population and how survival of symptomatic flies differs compared to asymptomatic flies with a latent variable modeling approach. For each individual fly in the population there is a latent state, which describes that individual's daily infection status. The number of symptomatic individuals is expected to change over time so a variation of a susceptible-infected model (SI model) was used to define the latent status of individuals in the population. Estimated survival parameters within the model show a lot of overlap, as do the average lifetimes of individuals with the largest amounts of variation between replicates occurring in the Mock treatment. Importantly, despite the different analytical approach used by the latent variable mixture model, similar conclusions are drawn as the hierarchical model regarding the effects of the interactions between viruses being non-additive.

Our results show that DCV and DXV act antagonistically upon pathogenesis within small, closed populations of *D. melanogaster*. There is no evidence of additive effects between the viruses in regards to the population demographic property of mortality rates in coinfection. It is likely that both viruses are competing for necessary resources within a host as they infect similar tissue types (fat, body, digestive tract) which may be associated with oral/fecal routes of infection (Xu & Cherry 2013). Additionally, single infection of DCV appears to eliminate variation in conditional mortality probability without strongly affecting the rates of mortality. DXV follows similarly in eliminating variation, but the effect is not immediate as with DCV. While there are clear trends in the data, one major drawback in this study is that it is not possible to confirm the infection status of an individual deceased fly. Assumptions can be made regarding the infection status of individual flies, but there is no

empirical data to definitively support or deny those assumptions as flies could not be collected and assayed at the time of mortality without disturbing the experimental setup. It is therefore quite difficult to state the efficacy with which DCV and DXV act upon host mortality and how many mortality events within a given treatment are truly associated directly with infection. This issue is a focal point of the latent variable mixture model being prepared by Ferguson et al. Further studies should be done to quantify the proportion of mortality events within a treatment population that can be definitively associated with infection both by assay and by reduction or elucidation of the mechanisms of the prevalent unknown risk factors for mortality.

TABLES

Table 1.1. Likelihood ratio tests for Mock vs. viral treatments across all time intervals. There is strong support for differing trends in mortality between Mock and DCV, weak but non-significant evidence for a difference between Mock and DXV, and no evidence for a difference between the Mock and COI. This implies the strongest response to infection is caused by DCV.

Comparison	Test Statistic	P-value
Mock v DCV	9.1957	0.0101
Mock v DXV	5.1595	0.0758
Mock v COI	3.9752	0.1370

Table 1.2. Likelihood ratio tests between viral treatments across all time intervals. There is no evidence for a difference in trends between DCV and DXV treatments or between DXV and COI treatments. There is evidence for a stronger effect of viral infection in DCV infected flies between the DCV and COI treatments.

Comparison	Test Statistic	P-value
DCV v DXV	3.5152	0.1724
DCV v COI	6.5089	0.0386
DXV v COI	2.3433	0.3098

Table 1.3. Parameter estimates for fits of model to individual treatments. μ and ϕ are transformations of controlling parameters α and β. μ_1 and μ_2 represent expected mean values across beta distributions 1 and 2 respectively. Distribution 1 refers to the initial time-period comprised of weeks 1 and 2. Distribution 2 refers to the second time-period comprised of weeks 3 through 5. Φ_1 and Φ_2 are representative of variance among replicates for a treatment within distributions 1 and 2 respectively. DCV exhibits a slightly higher distribution mean than Mock or other viral treatments. DCV also shows greatly diminished variances when compared to the Mock line across both time-periods. DXV also shows diminished variance resembling the DCV treatment, but only in the latter time-period. In the first time-period DXV more closely resembles Mock and COI treatments both in means and variance.

Treatment	μ_1	μ_2	ϕ_1	ϕ_2
Mock	0.0358	0.2441	0.0463	0.0929
DCV	0.1003	0.2131	$8.4e^{-8}$	$8.5e^{-8}$
DXV	0.0672	0.2735	0.0698	$1.6e^{-7}$
COI	0.0677	0.3090	0.0162	0.0573

FIGURES

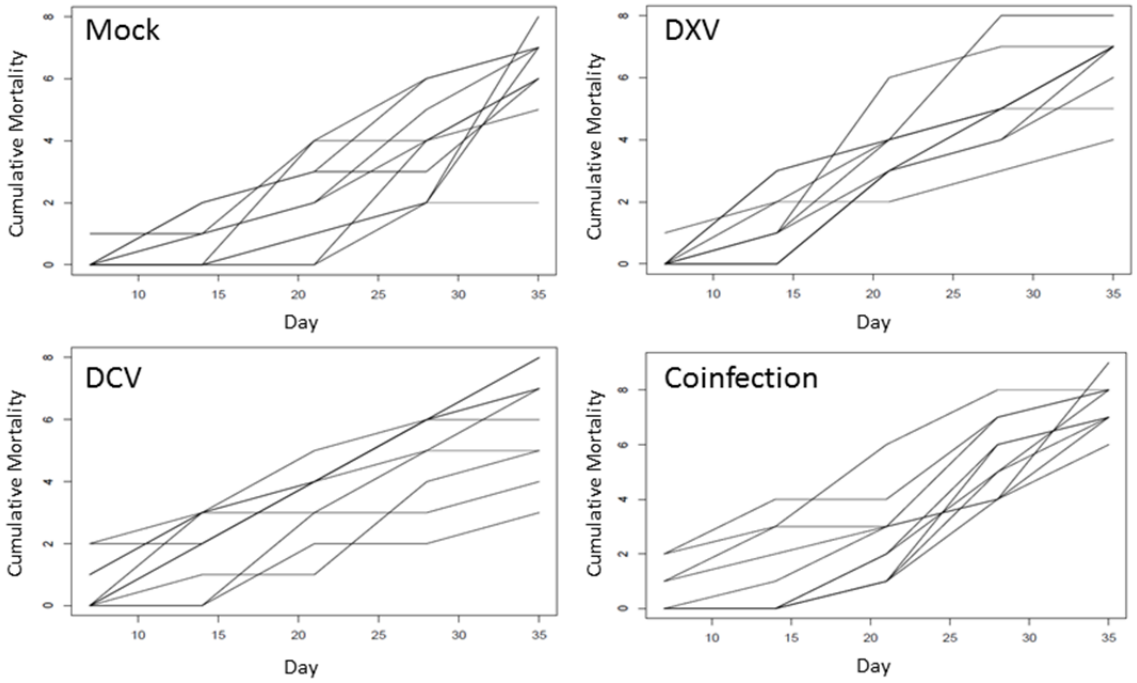

Fig. 1.1. Cumulative mortality over time for individual replicates within a treatment. Each line in a plot is representative of a single replicate vial beginning with 10 flies total.

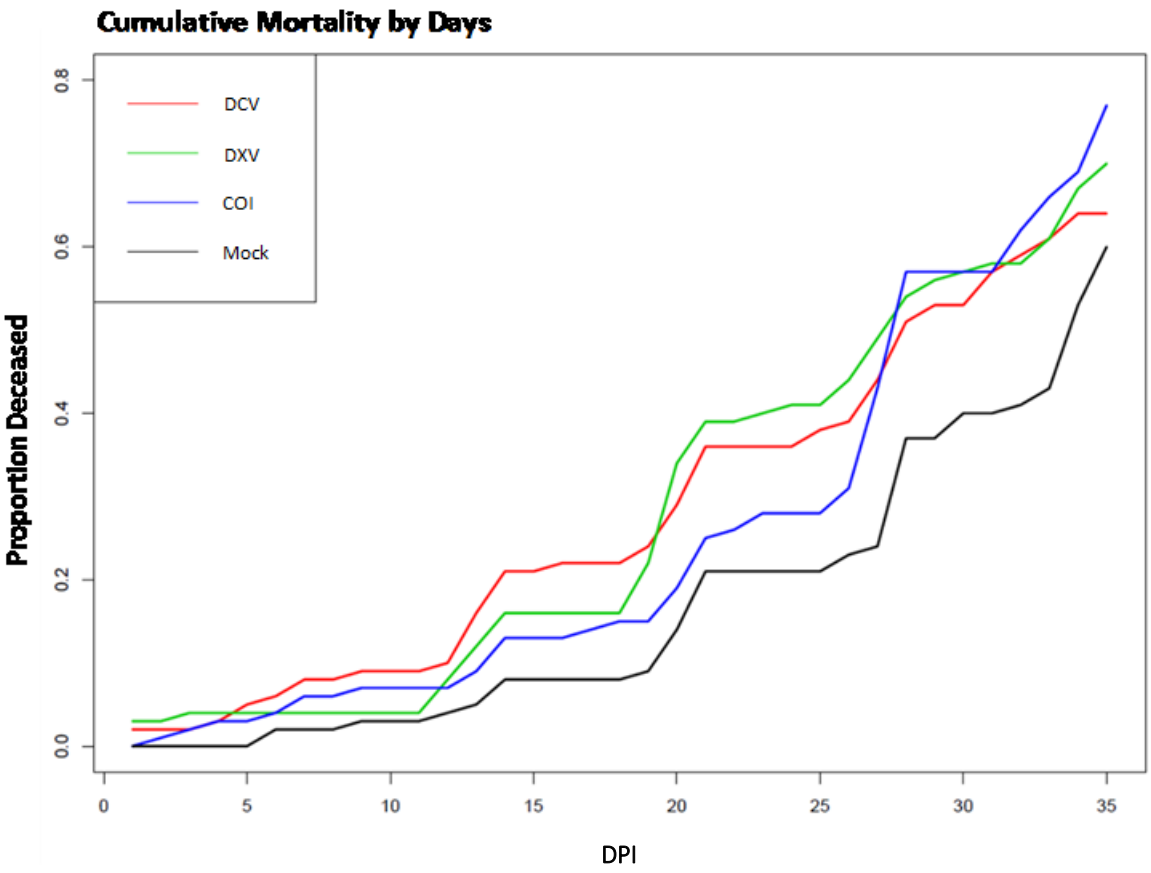

Fig. 1.2. Proportions of cumulative mortality for each treatment over 35-days post infection (DPI). Inset key shows color and treatment correlation where DCV is represented in red, DXV in green, COI in blue, and Mock in black. Mortality counts are representative of cumulative mortality across all replicate vials for a given treatment at each time point.

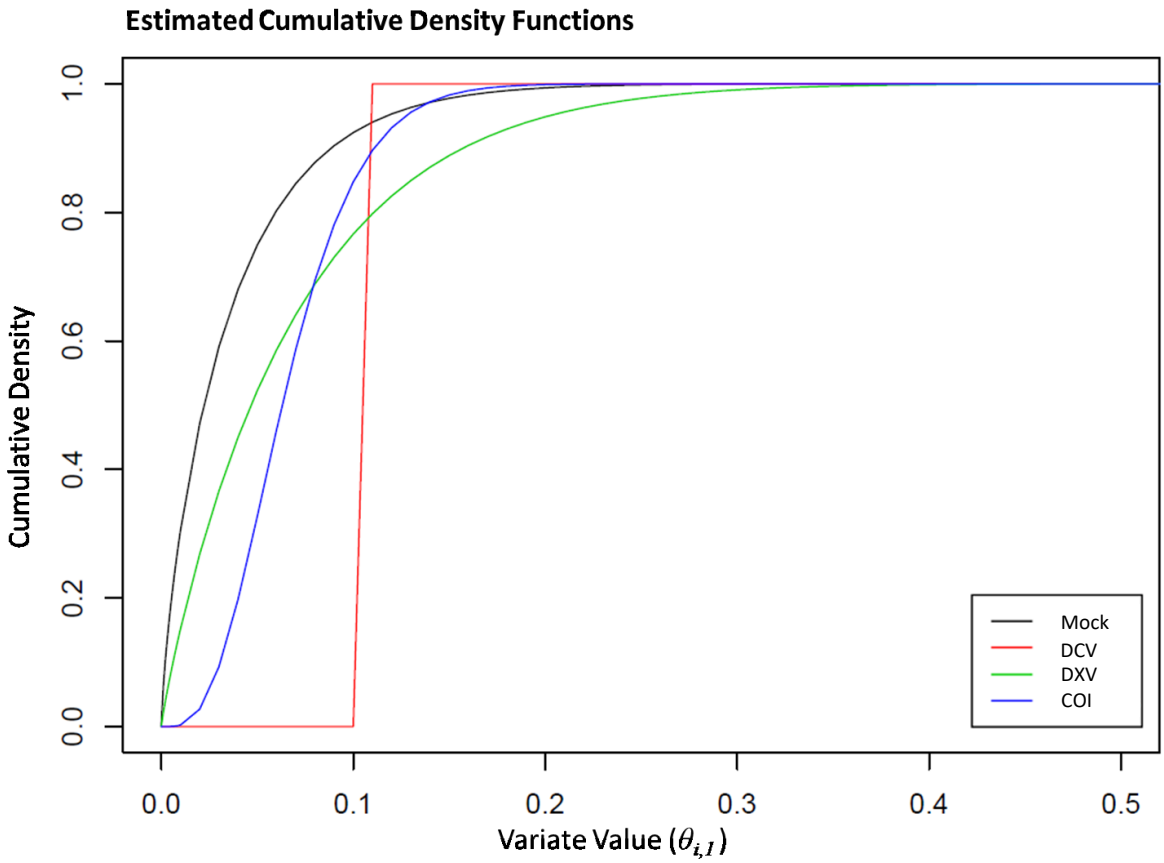

Fig. 1.3. Estimated cumulative density functions for all treatments in time-period 1 (weeks 1 &2). Inset key shows color and treatment correlation where DCV is represented in red, DXV in green, COI in blue, and Mock in black. The probability of $\theta_{i,1}$ for a given treatment ≤ to the value on the x-axis is equal to the corresponding cumulative density value on the y-axis at that point. Therefore, the distribution for the conditional probability of mortality for a treatment is between the values on the x-axis where the curve falls between cumulative density = 0.0 and 1.0. The more variable a distribution, the shallower the curve. Inversely, the sharper the curve, the less variable a distribution. There is a very limited distribution for the conditional probability of mortality ($\theta_{i,1}$) in the DCV treatment compared to the Mock and other viral treatments; roughly between 0.1 and 0.13. DCV also has the highest minimum value of conditional probability for mortality.

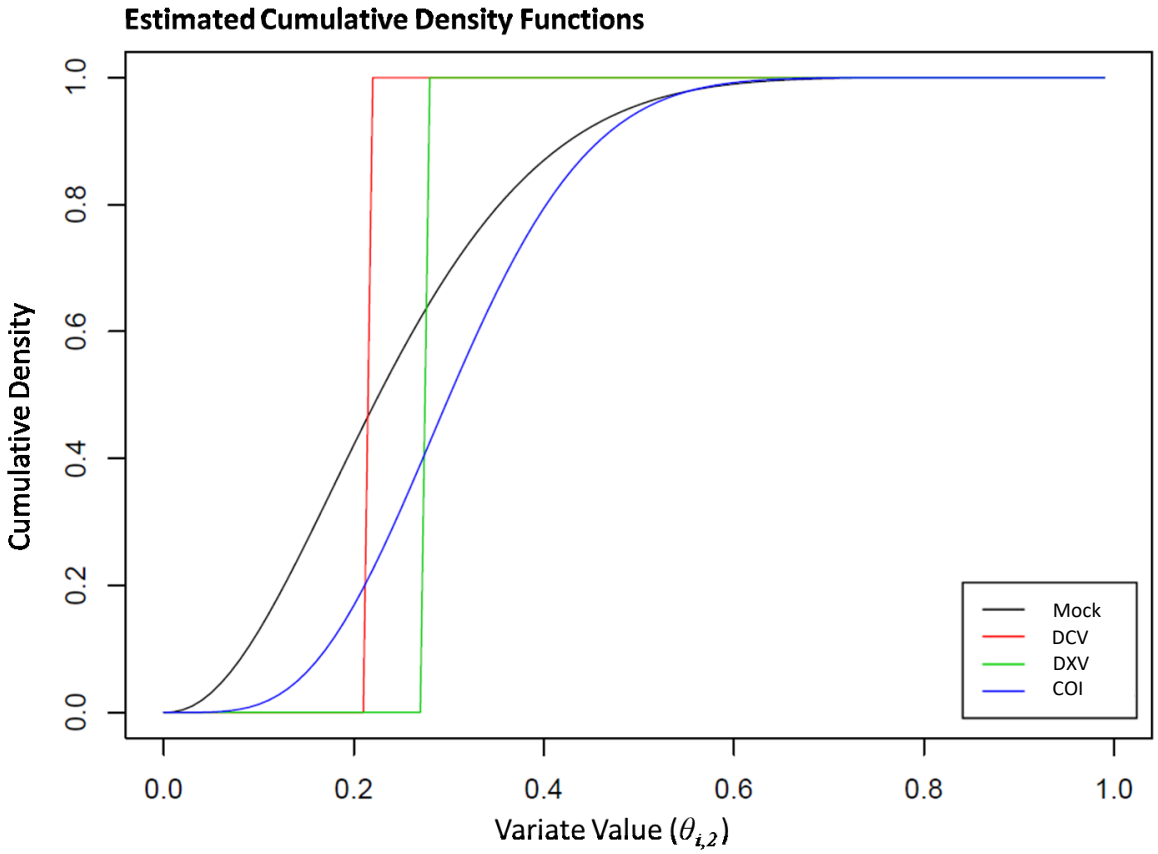

Fig. 1.4. Estimated cumulative density functions for all treatments in time-period 2 (weeks 3-5). Inset key shows color and treatment correlation where DCV is represented in red, DXV in green, COI in blue, and Mock in black. The probability of $\theta_{i,2}$ for a given treatment ≤ to the value on the x-axis is equal to the corresponding cumulative density value on the y-axis at that point. Therefore, the distribution for the conditional probability of mortality for a treatment is between the values on the x-axis where the curve falls between cumulative density = 0.0 and 1.0. The more variable a distribution, the shallower the curve. Inversely, the sharper the curve, the less variable a distribution. There is a very limited distribution for the conditional probability of mortality ($\theta_{i,2}$) in both the DCV and the DXV treatment compared to the Mock and COI treatment.

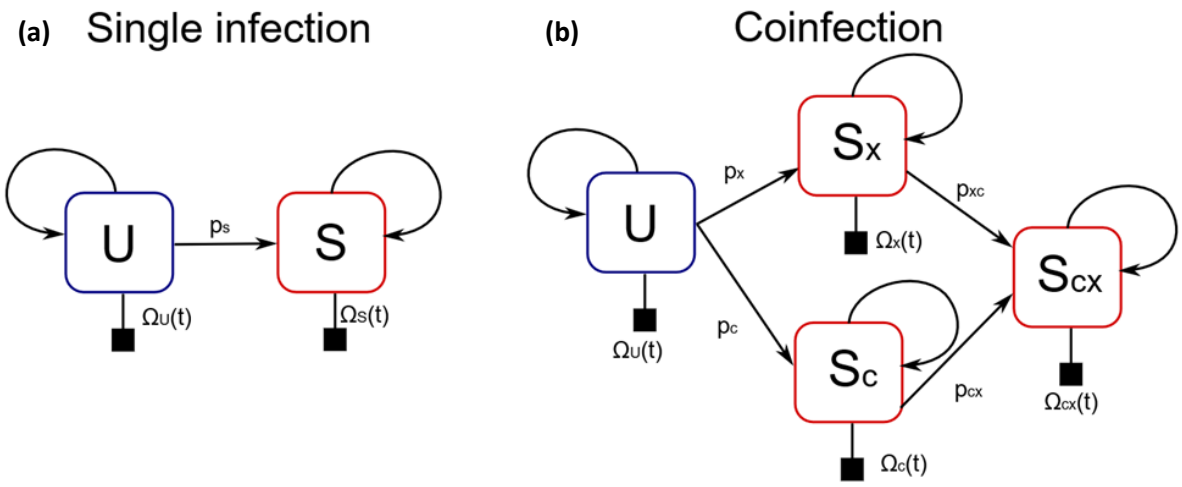

Fig. 1.5. *Adapted from Ferguson et al. (in prep).* Schematic of latent variable mixture model for (a) single infection and (b) coinfection events. Flies are classified as being asymptomatic (U) or the symptomatic (S) for DXV (S_X), DCV (S_C) or Coinfection (S_{CX}). $\Omega_k(t)$ is the daily probability of death for type k and P_{ki} is the probability of an individual transitioning from one class (k)

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Chapter 2

EXPLORING VIRAL TRANSMISSION VARIABLES FOR A TRACTABLE MODEL OF VIRAL COINFECTION IN *DROSOPHILA MELANOGASTER*

ABSTRACT

Understanding the dynamics of pathogen transmission is a core component in modeling epidemiological events. Interactions between competing pathogens in a population may affect the dynamics of pathogen transmission by altering the likelihood of transmission or the severity of infection associated with disease transmission. Utilizing a system of *Drosophila melanogaster* and two associated viruses, we set a framework for exploring the effects of viral coinfection on transmission likelihood and the severity of infection associated with those viral transmission events. In triplicate, we analyzed small populations of flies acting as recipients (here referred to as sentinels) of viral transmission by exposure to a single infected donor across three viral treatments, Drosophila C virus (DCV), Drosophila X virus (DXV), and Coinfection (COI). Transmission was measured by quantitative reverse transcription PCR (RT-qPCR) of sentinel flies. Likelihood ratio tests were used to determine differences between transmission probabilities. Infection severity for confirmed transmission events was determined by generating estimates of parameters acting as a proxy for starting viral load. These estimates were based on raw RT-qPCR fluorescence data that was analyzed through a Bayesian hierarchical model. Those estimates were then set as response variables in a linear regression model. We find that DXV transmission is completely nullified by coinfection events. However, we find no evidence for altered DCV transmission probability between single and coinfection events. Additionally, deceased DCV carrying donors had a greater likelihood of transmission to other adult flies than live donors. This is true in both the DCV and COI treatments. This effect was not exhibited in the DXV treatments. Although there is no evidence for a difference in DCV transmission probability between single and coinfected populations, we find weak, but non-significant support for higher initial viral loads of DCV in single infection populations. Results from the combined

analyses suggest an interfering effect between viruses on transmission dynamics, although more data will be required to draw a definitive conclusion.

INTRODUCTION

The rate at which susceptible individuals are exposed to a changing pool of viruses in any natural system is likely to be higher than individual viral clearance rates. This in turn, provides ample opportunity for viral coinfection events, which have been shown to significantly alter viral dynamics in many systems (Pournik et al. 2013, Mulcahy et al. 2011, Tao et al. 2015). These altered dynamics may also be associated with changes in pathogenesis through infection duration, intensity, and transmission (Gonzalez et al. 2016, Susi et al. 2015). The factors that alter the ability of a pathogen to replicate within host may in turn affect pathogenic shedding patterns (Pinky & Dobrovolny 2016). Between hosts, patterns of transmission may vary based on host behaviors and pathogenic routes of transmission (e.g., direct contact, airborne, oral/fecal, waterborne, etc.) (Aiello et al. 2016). The rates and modes of transmission are key components in studying and modeling the epidemiology of any viral infection and should be considered in coinfection events. However, studies on modeling transmission are often limited by parametric and structural uncertainties (Foss et al. 2009). Many of these uncertainties may be exacerbated in coinfection events, stemming from a lack of knowledge regarding the true underlying values for variables in transmission events such as initial titer, rate of infection, and severity of infection. While many epidemiological models are very focused and dependent on the dynamics of a specific disease it is still valuable to generate models to understand how pathogens interact with each other to affect host population dynamics in a more generalizable system.

Arboviruses pose major concerns to human related disease prevention efforts, impacting a wide range of systems from vertebrates to plants (Blanc & Gutiérrez 2015). A tractable invertebrate model for viral coinfection may be leveraged for expanding on the expectations of arbovirus dynamics. The present study explores the effects of viral coinfection on viral transmission in an empirical model system of *Drosophila* and two associated viruses, Drosophila C virus (DCV) and Drosophila X virus (DXV). These viruses

exhibit variation in pathogenesis, trigger different host immune responses, and have been shown to alter population demographic properties such as mortality and fecundity in coinfection versus single infection events (Xu & Cherry 2014, Ferguson et al. *In prep*. *See Chapter 1*).

Preliminary work in this system indicates that DCV and DXV are shed by infected hosts but require time to accumulate to infectious levels in the environment, or are rapidly shed and picked up but require time to induce lethal infection within the host. In either case, transmission seems to occur by viral shedding and indirect transmission from the environment rather than from direct contact between flies. Additionally, in 2015, Longdon et al. demonstrated that deceased adult donors are much more likely to transmit DCV to larvae than live adult donors. This all suggests that within this empirical system, it is pertinent to explore differences in rates of transmission between adults from both live and deceased infected donors.

This study is aimed at developing a framework for determining how the transmission rates and infection severity of DCV and DXV are altered by the presence of both viruses within a shared host. Likelihoods for transmission across treatments were determined by the proportion of susceptible individuals in small, experimental populations that develop a viral infection. These susceptible individuals acted as sentinels for viral presence and are exposed to viral treatments only through a visually distinguishable single infected donor (*Drosophila* white-eye mutant) within the population. The severity of each infection associated with a transmission event was then determined by comparison of estimates of viral amplicon expression relative to the constitutive expression of *Drosophila* ribosomal housekeeping gene *RpL32* (Chrostek et al. 2013).

MATERIALS & METHODS

Fly and virus stocks

Oregon R WT^{+} strain of *Drosophila melanogaster* were used for all sentinel group flies. White-eye mutant flies with the white-eye mutation W[1118] were acquired from the Bloomington Drosophila Stock Center, Bloomington, IN, and account for all white-eye female donors (stock No. 3605). Both wildtype and white-eye fly stocks were treated for

viral and bacterial infection via egg bleaching and tetracycline (Merkling & van Rij 2015). Flies were incubated in a Genesee Scientific I Drosophila incubator, model: 59-400LH on 12-hour night/day cycles at 25°C and at 65 percent relative humidity. Fly stocks were separated into four groups reared in weekly increments. Each group was reset monthly by the random sorting of five male and five female flies per vial, from the current two-week old stock population to form the new group. All flies utilized in experiments were age controlled from the same stock group. The age-controlled flies were placed into vials of 30 flies with roughly even sex distribution for sentinel groups and only females for white-eye donor groups. All experimental flies were kept in the same conditions as the stock lines described above.

Two viruses were utilized in this study, Drosophila C virus (DCV) and Drosophila X virus (DXV). DCV is a single stranded RNA virus, 9,264 base pairs in length, and a member of the family *Dicistroviridae* family. We used the Charolles strain of DCV that was isolated from a laboratory population of *Drosophila* in 1972, (Jousset et al. 1972) and that we obtained from Dr. Luis Teixeira (Instituto Gulbenkian de Ciencia). DXV is a bi-segmented, double-stranded RNA virus, 6,603 base pairs in length, and a member of the family *Birnaviridae* (Teninges et al. 1979, Nagy & Dobos 1984). DXV was originally isolated in 1979 as a contaminant in studies involving Sigma virus in *Drosophila melanogaster* (Teninges et al. 1979). We obtained our DXV stock from Dr. Louisa Wu (University of Maryland). Both DCV and DXV were cultured in Schneider's Drosophila Line 2 (S2) cells and titrated to a tissue culture 50% infectious dose ($TCID_{50}$) of $2x10^9$ units/mL. This is the endpoint dilution of virus required to cause cytopathic effects in 50% of inoculated cell cultures. Viral stocks were kept in 50µL aliquots at -80ºC.

Experimental design

Determination of how the infection status (uninfected, DCV+, DXV+, or coinfected) of a host fly affects the rate and severity of viral transmission by DCV and DXV was conducted using two groups of flies. Female flies of a mutant strain of white-eye *D. melanogaster* (visibly distinguishable from the red-eye WT^+ flies) were used as viral donors and injected with a predetermined volume of viral solution as described below. Flies of a wildtype strain of *D. melanogaster* acted as sentinels, i.e. uninfected flies to be tested for

viral presence after being exposed to infected donors. All experimental flies were controlled for age effects by dumping of stocks and using flies emerged within a 24-hour period and subsequently aged three days to sexual maturity. Following injection, donors were immediately housed with five male and five female sentinel flies in fresh food vials. Any vials with donors found deceased within a 24-hour period were discarded as donors were assumed to have died due to stress or injury induced by the injection process. When found beyond the initial 24-hour period, deceased donors were removed and immediately transferred to a new vial with a new set of sentinel flies. This allowed for comparison of viral transmission by live and deceased donors using the same individual donor. Any sentinels housed with a live donor were removed by anesthetization on ice and transferred to new vials to be kept in isolation for an additional three days at 25°C to allow for within host viral replication. This three-day incubation period is meant to increase the efficacy of subsequent viral detection assays via qRT-PCR if transmission and infection have occurred (Longdon et al. 2015). New sentinel groups were housed with deceased donors for the same three-day duration of exposure as live donor exposed sentinels. Following this period, sentinels were collected and isolated in the same manner as the first sentinel group from live transmission. Collected flies, including donors, were frozen, and stored at -80ºC to preserve both host and viral RNA for a later viral assay.

Viral injection of donor flies

Age controlled 3-day old white-eye female donors were injected with a Drummond Scientific Nanoject-II (Cat. No. 3-00-204) microinjector. We used 3.5 inch capillary needles that were pulled on a Stutter Instrument P-97 Flaming/Brown type micropipette puller and broken by hand under a dissecting scope to approximately 0.5µm in diameter. Viruses were diluted from stock aliquots at a $TCID_{50}$ of $2x10^9$ units/mL by a factor of 1:100 in S2 cell media to a $TCID_{50}$ of $2x10^7$ units/mL. Forty-six nanoliters of viral solution was injected per fly at a rate of 46.0nL/sec for approximately 920 $TCID_{50}$ per injection. To minimize the variance in effect of the inoculation process on the flies, all injections were intra-abdominal with the injection site at the ventral-dorsal surface junction of the upper right abdomen (Merkling & van Rij 2015). Due to symptoms of CO_2 sensitivity induced by DXV (Teninges

et al. 1979), flies across all treatments were anesthetized on ice and injected on cold blocks (stored at -20^{o}C) rather than on CO_2 pads.

Viral assay

Total RNA was extracted from individual flies using an optimized Trizol RNA extraction protocol (Life Technologies Cat. No. 15596026) and treated with DNase I using the TURBO DNA-free, Kit TURBO DNase Treatment and Removal Reagents (Life Technologies Cat. No. AM1907) following the standard protocol for 50µL suspensions of female RNA and 30µL suspensions of male RNA. Flies were assayed for viral presence using the Luna Universal One-Step RT-qPCR Kit optimized for dye-based real-time quantification with SYBR green using 10µL reactions on 35ng of total sample RNA per reaction. Cycle program followed standard protocol according to the Luna Universal One-Step RT-qPCR Kit manual for fast ramp speeds on an Applied Biosystems StepOnePlus machine. Each sample and control was run in duplicate on 96-well plates in singleplex reactions.

Viral primer sequences used were as follows:
DCV forward: 5'-GCAGTTGAATCTCCCCGTGA-3' &
DCV reverse: 5'-AGAGCAACATCTGACGTGCA-3'.
DXV forward: 5'-CGCAGGCTTATTTCGCACTC-3' &
DXV reverse: 5'-TCACCCTCCTCTCTCTGACG-3' (Gonzalez-Gonzalez et al. in prep).
The primer for the constitutively expressed reference was
RpL32 forward: 5'-CCGCTTCAAGGGACAGTATC-3' &
RpL32 reverse 5'-CAATCTCCTTGCGCTTCTTG-3'.
RNA extracted from wild-type uninfected controls was used to set the baseline levels transcripts for the housekeeping reference gene RpL32.

Analysis

Data from three sets of experimental flies (donor, live exposure sentinels, deceased exposure sentinels) for each treatment (DCV, DXV, COI) were prepared and used in analysis for both transmission occurrence and severity of infection. Transmission events were determined using a combination of the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001) and

manual confirmation of plate by plate data confirming amplification of the desired product through melting curves where the T_m for DCV ≈ 80ºC, DXV ≈ 85ºC, and for endogenous control *RpL32* ≈ 89ºC.

Cycle thresholds (C_t) were set as the cycle in which the change in fluorescence above baseline (ΔRn) is greater than or equal to 0.8 for all treatments. Mean C_t was determined for each sample by averaging across duplicates for both target of interest and the reference gene *RpL32*. These values were compared to negative controls for both the viral target and *RpL32* to obtain control and experimental ΔC_t values and sample $\Delta\Delta C_t$ values following *equations 2.1* and *2.2* respectively:

$$\Delta C_t = C_t \text{ (Viral Target)} - C_t \text{ (}RpL32\text{)} \qquad \textbf{(2.1)}$$

$$\Delta\Delta C_t = \Delta C_t \text{ (Viral Target)} - \Delta C_t \text{ (}RpL32\text{)} \qquad \textbf{(2.2)}$$

Values for $2^{-\Delta\Delta Ct}$ were then calculated from $\Delta\Delta C_t$ values as representations of viral expression in each sample relative to a negative baseline. Flies with values of $2^{-\Delta\Delta Ct}$ near 0.01 were considered infected and confirmed on a case by case basis by observation of amplification and melting curves in Applied Biosystems StepOne Software v2.3.

Comparison of transmission likelihood between sentinels across or within treatments was conducted by treating each donor fly as a binomial for the occurrence or absence of viral target transmission. The binomial sample size was equal to the number of sentinels exposed to the donor in a vial. This led to 3 binomials for each treatment/viral target combination for either live or deceased donor exposure.

Severity of infection in transmission events were represented by estimates of starting viral target fluorescence (μ_o). Raw qPCR fluorescence data were truncated to 30 PCR cycles to minimize background noise and then analyzed using a Bayesian hierarchical model implemented through the R package RStan (Ridenhour et al. 2017, Hoffman & Gelman 2014). This model uses a Hamiltonian Monte Carlo (HMC) algorithm for sampling posterior distributions of model parameters. We ran the sampler for 4 independent chains of 2,000

generations with the first half discarded as a burn-in period. This is an adapted model from Ridenhour et al. (2017) in which, within wells, the model follows a time dependent solution

$$x(t) = \frac{x_0 + y_0}{1 + \frac{x_0}{y_0} e^{-k(x_0+y_0)t}} \quad \textbf{(2.3)}$$

Where x_o represents the starting RNA concentration, y_o is the starting primer concentration, k is the rate of reaction and t is the number of PCR cycles. We estimate the quantity x_o in order to draw conclusions about initial viral loads in the sample flies relative to the housekeeping RNA for *RpL32*. Mean estimates for x_o within the distributions generated by the HMC algorithm are represented by μ_o and were utilized in conjunction with factorial demographic data as the response in a generalized linear model. The linear model followed the structure:

$$\mu_o \sim \text{Treatment} + \text{Sex} + \text{Live or Deceased exposure}$$

with an offset equal to the log of $\mu_{o,RpL32}$ (μ_o estimates for reference gene *RpL32*).

RESULTS

Transmission likelihood

Transmission events between donors and sentinels are marked by a relative target expression fold change ≥ 0.007. Observation of raw counts of transmission events between experimental groups shows that transmission occurred more frequently for DCV than for DXV across both single and coinfection treatments (*fig. 2.1*). Additionally, it was observed that DXV was not transmitted by either live or deceased coinfected donors within the 3 days of exposure time. Transmission was more common in deceased exposure sentinel groups than live exposure groups across all treatments, accounting for ~78% (35/45 events) of the total transmission events amongst the three treatments.

Likelihood ratio tests for DCV transmission between live and deceased donor groups within both the DCV and COI treatments show a significant increase in the probability of transmission within deceased donor exposure replicates (DCV: *T* = 14.712, P = 1.3e-4*; COI:C: *T* = 25.189, P = 5.197e-7*) (*table 2.1*). However, there is no evidence for differing probabilities of transmission between the two treatments in total or between the subsets of live and deceased exposure group sentinels (*table 2.1*).

Likelihood ratio tests for DXV transmission between live and deceased donor groups within the single infection treatment were not appreciably different (*table 2.2*). Due to the absence of DXV transmission from coinfected donors, model estimates for transmission of DXV involving coinfected groups are all equal to zero. Although no values can be generated from the zero estimates there exists an inherent significant difference between DXV transmission across single and coinfection treatments. This is because the probabilities of DXV transmission from either the deceased or live donor group are non-zero. Conversely, there is no difference in the likelihood of DXV transmission between COI live and deceased exposure groups as they are identical.

Likelihood ratio tests between the DCV and DXV treatment groups show no difference in the likelihood of transmission among live donor exposure groups. However, there is clear evidence for an increase in the likelihood of transmission within the deceased donor exposure group for DCV ($T^* = 9.770, P = 0.002$). This effect is strong enough to drive a significant difference in the overall probability of transmission between the treatments in general ($T^* = 6.320, P = 0.012$) (*table 2.3*).

Severity of infection

Sentinel flies positive for infection and therefore associated with transmission were further processed by analysis of the raw qPCR fluorescence data. Ratios of fluorescence between viral targets and the reference gene *RpL32* were generated on a well-by-well basis and used to develop estimates of baseline fluorescence (μ_o) for each individual sample. Values of μ_o act as a proxy for the viral load within flies at the time of collection, with higher baseline fluorescence associated with higher concentrations of viral target RNA. The probabilities of μ_o were estimated as the response variable in a generalized linear model. These estimates were applied only to flies considered positive for viral infection, as base fluorescence in noninfected flies was effectively zero.

Model coefficient estimates provided in *table 2.4* show that none of the fixed effects used in the model (Treatment (DCV or COI), Sex, Live or Deceased donor exposure) had a significant effect on the response μ_o. While not significant, there is weak evidence for a positive effect on the response μ_o by single infection DCV treatment, with the largest fixed effect coefficient falling outside of a single standard deviation from 0 ($Est. = 0.722, T^* =$

1.575, P = 0.12). This effect is represented graphically in *figure 2.2* with the mean estimate of starting fluorescence for infected DCV treatment sentinels sitting well above the mean estimate starting fluorescence of the infected sentinels in the COI treatment. DXV infections were not analyzed in comparison to the COI treatment manner due to the lack of DXV transmission/infection events in the COI treatment.

DISCUSSION

Coupling of the likelihood ratio tests and the raw counts for transmission indicate that DCV is more effectively transmitted between adult flies by a deceased donor within both the single and coinfection treatments. This is likely due to an oral/fecal route of transmission for DCV in which horizontal transmission of the virus occurs by shedding onto and ingestion from the nutritive media (Gomariz-Zilber & Poras 1995). This result is also in line with work done by Longdon et al. (2015) which showed that deceased adult donors transmit DCV to larvae at a much higher rate than live adult donors. DXV does not exhibit this same trend and shows no statistically significant difference in transmission rates when comparing live and deceased donors. DXV also exhibits lower infectivity than DCV overall, in both single and coinfection treatments. It is possible that DXV has a longer natural course of infection and that three days of exposure to a potential viral donor is not a sufficient time span for viral shedding, infection, and viral replication.

When the likelihood of transmission events is compared across treatments there is a clear negative effect on the transmission of DXV by coinfection events in that DXV is not transmitted at all when donors are also infected with DCV. The likelihood of DCV transmission on the other hand, is not appreciably different between the single and coinfected groups. Within the coinfected donors DCV may be outcompeting DXV for necessary resources or is better suited to avoiding host immune responses. The DCV genome has been documented to encode for an RNAi suppressor, which is often regarded as the primary means of antiviral defense in the *Drosophila* innate immune system (van Rij et al. 2006). Additionally, the RNAi response in *Drosophila* is not thought to operate systemically, but rather on a cell autonomous level (Van Roessel et al. 2002, Zambon et al. 2006). Coupled together, it is possible that DCV is better equipped for initial exploitation of host cells, and then within those cells, suppressing the host immune response. The same

experiment conducted in the present study could be done substituting experimental flies with knockout flies that do not encode for the RNAi associated proteins Dicer and Argonaute (Wang et al. 2006).

Estimates of starting fluorescence in single infection treatment sentinels show weak support for a trend of increased viral loads present in sentinels that received an infection over the course of the experiment in DCV single infection. This suggests that, although DCV did not exhibit differing rates of transmission in single infection when compared to COI, that when transmission does occur, the resulting infection is more severe. No other demographic effects included in the linear model (sex, live or deceased donor exposure) show any evidence for an effect on viral loads. This analysis was not able to be conducted on DXV in this study due to the lack of DXV transmission events in the samples analyzed. Ideally, a mixed model could be applied to the data set in which donors associated with experimental groups act as random effects in the model. However, within the scope of this exploratory experiment the data are not sufficient to definitively uncover these suggested trends associated with estimated initial fluorescence.

Our data suggest overall non-additive effects of coinfection on transmission dynamics in our *Drosophila* system and provide evidence for an antagonistic effect on the transmission dynamics of DCV and DXV in small populations exposed to coinfected donors. There effects are exhibited by means of reduced transmission in DXV and reduced infection severity in DCV, although the sample sizes of the study are limited. Increased sampling is a necessary step moving forward to expand on these conclusions and generate increased statistical and modeling power.

TABLES

Table 2.1. Likelihood ratio tests for transmission events between sentinel groups exposed to live and deceased donors with DCV as the target of interest. Live donor groups are designated by "live" and deceased donor groups by "dead". There is a significant difference between transmission likelihood of DCV between sentinels exposed to deceased donors and live donors. This trend is present in both the single and coinfected treatments. There is no support for a difference in the likelihood of DCV transmission across single or coinfected treatments with the same exposure type (live or deceased exposure). The final row is a comparison of the likelihood of DCV transmission events between all sentinels of the COI and DCV treatment groups and exhibits no evidence for a difference in transmission likelihood between the two treatments.

Comparison	Test Statistic	P-value
DCV live v DCV dead	14.712	1.300e-4*
DCV live v COI live	0.000	1.000
DCV dead v COI dead	1.724	0.189
COI:C live v COI:C dead	25.189	5.197e-07*
DCV v COI	0.959	0.327

Table 2.2. Likelihood ratio tests for transmission events between sentinel groups exposed to live and deceased donors with DXV as the target of interest. Live donor groups are designated by "live" and deceased donor groups by "dead". There is not a significant difference between the likelihood of DXV transmission between sentinels exposed to deceased donors and live donors in either the single or coinfected treatments. NaN (Not a number) values are present in comparisons of COI treatments due to the complete lack of DXV transmission. This causes estimates for transmission of DXV in COI treatments to be equal to zero. All comparisons between treatments COI and DXV are therefore inherently significantly different.

Comparison	Test Statistic	P-value
DXV live v DXV dead	0.541	0.462
DXV live v COI live	NaN	NaN*
DXV dead v COI dead	NaN	NaN*
COI:X live v COI:X dead	NaN	NaN
DXV v COI	NaN	NaN*

Table 2.3. Likelihood ratio tests between treatments DCV and DXV for transmission events. Live donor exposure groups are designated by "live" and deceased donor exposure groups by "dead". There is a significant difference between the occurrence of transmission events between DCV and DXV overall, as well as between the deceased exposure groups but not between the live exposure groups across treatments.

Comparison	Test Statistic	P-value
DCV v DXV	6.320	0.012*
DXV live v DCV live	0.005	0.941
DCV dead v DXV dead	9.770	0.002*

Table 2.4. Summary table of generalized linear model for starting fluorescence (μ_o) of DCV targets. No fixed effects exhibit a significant effect on μ_o. However, due to the small sample sizes available there does appear to be weak evidence for increased starting fluorescence values for DCV targets in single infection versus coinfection treated flies.

Coefficients	Estimate	Std. Error	T-value	P-value
Treatment (DCV)	0.722	0.458	1.575	0.120
Sex (M)	0.098	0.364	0.270	0.788
Donor Live	-0.594	0.904	-0.658	0.513

FIGURES

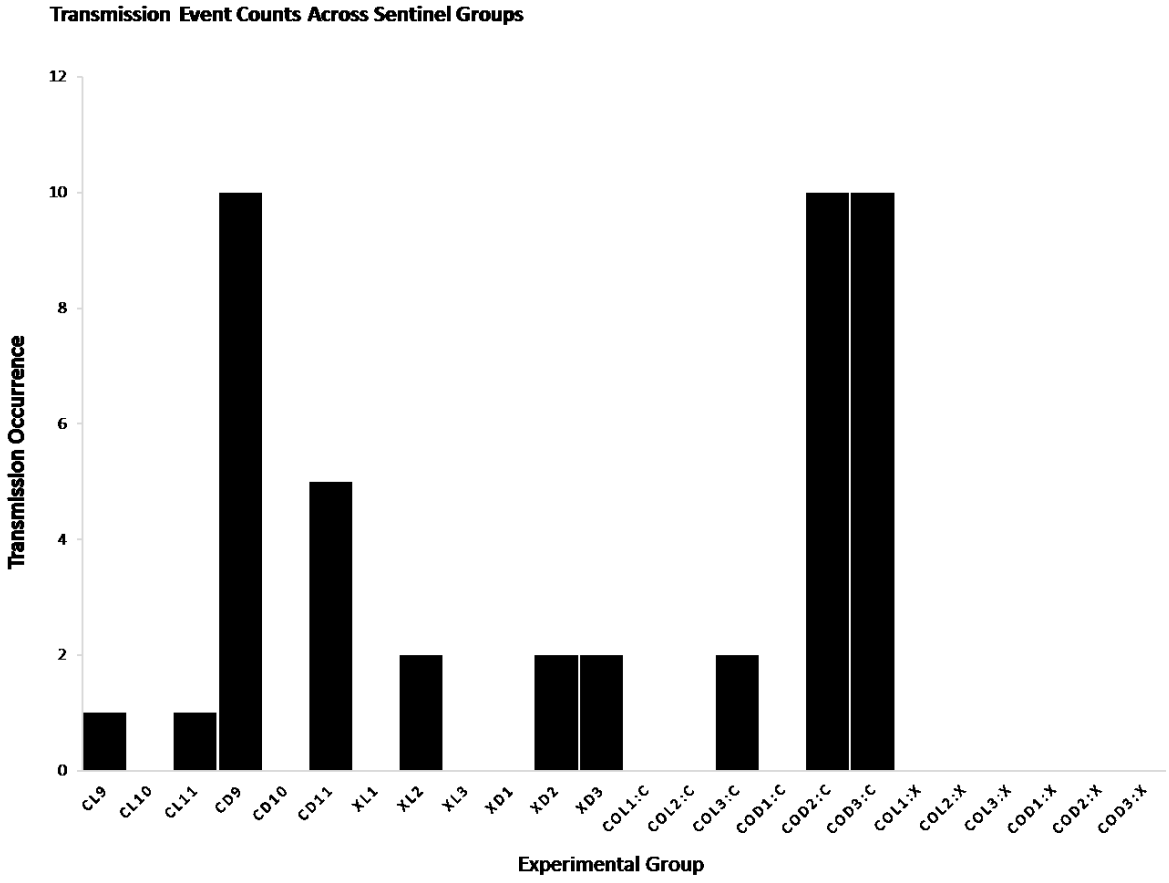

Fig. 2.1. Raw counts of transmission events occurring in each sentinel group. Group names begin with treatment designation (C = DCV, X = DXV, CO = COI) followed by donor mortality status (L = live, D = deceased) and finish with the replicate vial number. Note also that COI treatment vials are split between targeting for DCV and DXV as all qPCR reactions were singleplex. This distinction is represented by a colon followed by target virus. Most transmission events occurred for DCV in the deceased exposure sentinel groups for both mono- and coinfection treatments. There was no transmission of DXV in any coinfection treatment replicates of either live or deceased donor exposure.

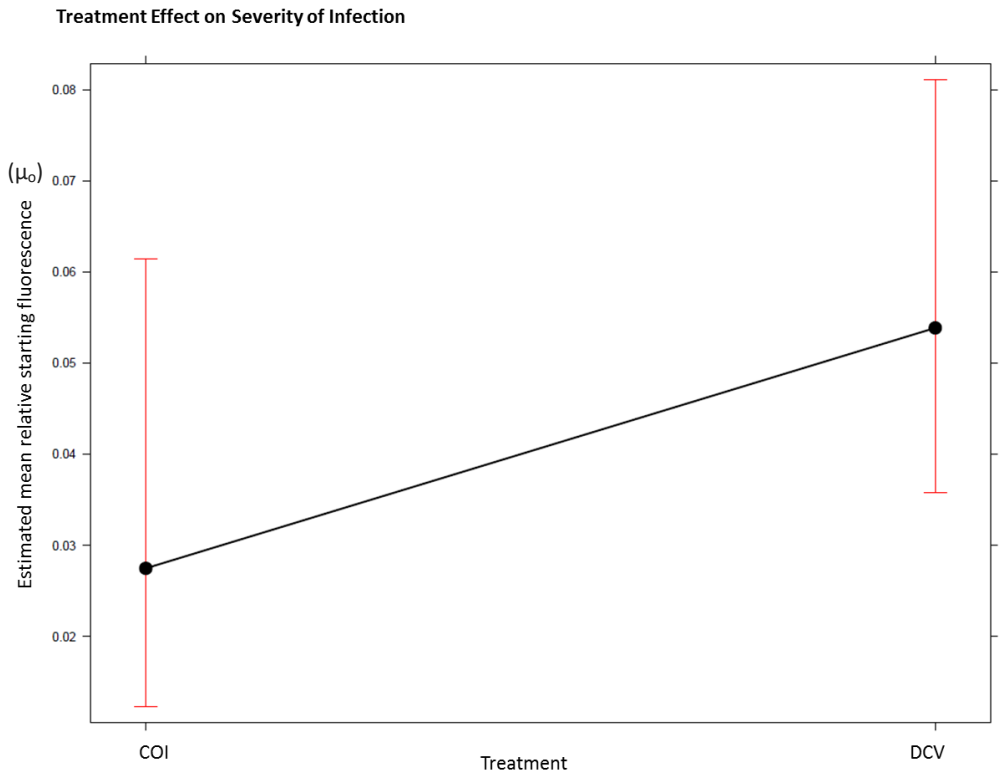

Fig. 2.2. Effect plot for estimated starting DCV fluorescence values (μ_o) between DCV and COI treatments. The estimated starting fluorescence values (μ_o) for the DCV treatment sentinels is higher than for sentinels in the COI treatment. Note that the average estimated μ_o for DCV positive controls = 0.0943.

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